

In the Claims

Amended Claims 1-6 and 8-11 are as follows, and appear in an *unmarked* claim listing in Appendix C.

1. (Currently amended, having been once previously amended) ~~A model for a mutually exclusive folding domain molecular switch including comprising a fusion protein comprising that includes at least one ubiquitin insert protein having an insert domain comprising a regulatory domain of ubiquitin lying between an amino terminal and a carboxyl terminal of said at least one insert protein, said insert domain being associated with a first quantity of free energy; and, a barnase target protein having at least one surface loop that begins at an alpha carbon of a first surface loop amino acid and terminates at an alpha carbon of a second surface loop amino acid, said at least one surface loop comprising a cytotoxic target domain of barnase of said target protein, said target domain being associated with a second quantity of free energy, wherein, said at least one insert protein is operatively inserted at a point within said at least one surface loop between said alpha carbon of said first surface loop amino acid and said alpha carbon of said second surface loop amino acid, such that an amino-carboxyl length extending between an alpha carbon of said amino terminal of said at least one insert protein and an alpha carbon of said carboxyl terminal of said at least one insert protein is at least two times greater than an $[\alpha\text{-carbon}-\alpha\text{-carbon}]$ length extending between said alpha carbon of said first surface loop amino acid and said alpha carbon of said second surface loop amino acid. A fusion protein comprising a ubiquitin insert protein having an insert regulatory domain lying between an amino terminal and a carboxyl terminal of the ubiquitin insert protein, the insert regulatory domain being associated with a first quantity of free energy; and, a barnase target protein having a surface loop that begins at an alpha carbon of an initial amino acid of the surface loop and terminates at an alpha carbon of a terminal amino acid of the surface loop, the surface loop comprising a cytotoxic target domain of the barnase target protein, the cytotoxic target domain being associated with a second quantity of free energy, wherein, the ubiquitin insert protein is inserted at a point within the surface loop between the alpha carbon of the initial amino acid of the surface loop and the~~

alpha carbon of the terminal amino acid of the surface loop, such that an amino-carboxyl length of the ubiquitin insert protein is at least two-times greater than an alpha-carbon-alpha-carbon length of the barnase target protein.

2. (Currently Amended) The fusion protein of claim 1, wherein ~~said the~~ insert regulatory domain exists in either a folded or unfolded conformation and ~~said the~~ target cytotoxic domain exists in either a folded or unfolded conformation, ~~said the~~ insert regulatory domain and ~~said the~~ target cytotoxic domain comprising a cooperative and reversible conformational equilibrium such that if ~~said the~~ insert regulatory domain is in its folded conformation, ~~said the~~ at least one target cytotoxic domain is in its unfolded conformation and vice versa.

3. (Currently Amended) The fusion protein of claim 2, wherein all or part of ~~said the~~ first quantity of free energy is made available to drive a folding of ~~said the~~ target cytotoxic domain from its unfolded conformation by means of a first controllable effector signal, and all or part of ~~said the~~ second quantity of free energy is made available to drive a folding of ~~said the~~ insert regulatory domain from its unfolded conformation by means of a second controllable effector signal.

4. (Currently Amended) The fusion protein of claim 3, wherein ~~said the~~ first controllable effector signal is selected from the group comprising ligand binding, pH, temperature, chemical denaturants, or mutations in either ~~said the~~ insert domain or ~~said the~~ target domain.

5. (Currently Amended) The fusion protein of claim 3, wherein ~~said the~~ second controllable effector signal is selected from the group comprising ligand binding, pH, temperature, chemical denaturants, or mutations in either ~~said the~~ insert domain or ~~said the~~ target domain.

6. (Currently Amended) The fusion protein of claim 2, wherein ~~said the~~ insert domain and ~~said the~~ target domain are disabled from simultaneously co-existing in their respective folded

conformations.

7. (Cancelled) The fusion protein of claim 2, wherein said insert domain and said target domain are disabled from simultaneously co-existing in their respective unfolded conformations.

8. (Currently Amended) The fusion protein of claim 2, wherein any excess of ~~said the~~ first quantity of free energy of ~~said the~~ insert regulatory domain that is not necessary to stabilize ~~said the~~ insert regulatory domain in its folded conformation is spontaneously transferred, through the structure of ~~said the~~ fusion protein, to ~~said the~~ target cytotoxic domain to unfold it from its folded conformation.

9. (Currently Amended) The fusion protein of claim 2, wherein any excess of ~~said the~~ second quantity of free energy of ~~said the~~ target cytotoxic domain that is not necessary to stabilize ~~said the~~ target cytotoxic domain in its folded conformation is spontaneously transferred, through the structure of ~~said the~~ fusion protein, to ~~said the~~ insert regulatory domain to unfold it from its folded conformation.

10. (Currently Amended) The fusion protein of claim 2, wherein ~~said the at least one~~ insert protein comprises human ubiquitin, ~~said the~~ insert regulatory domain comprises a regulatory domain of human ubiquitin, ~~said the~~ target protein comprises barnase, ~~said the at least one~~ target cytotoxic domain comprises a cytotoxic domain of barnase, ~~said the~~ amino-carboxyl length is about 38 Å, ~~said the first surface loop amino acid initial amino acid of the surface loop~~ comprises proline in the number 64 position ("Pro64"), ~~said the second surface loop amino acid terminal amino acid of the surface loop~~ comprises threonine in the number 70 position ("Thr70"), and ~~said the~~ alpha-carbon-alpha-carbon length is about 10.4 Å.

11. (Currently Amended) The fusion protein of claim 10 wherein ~~said the~~ regulatory domain of human ubiquitin and ~~said the~~ cytotoxic domain of barnase comprise a cooperative and reversible

conformational equilibrium, that may be determined by said the controllable first and second effector signals.

12. (Cancelled) A method for the production of a protein comprising the steps of:

- a. selecting a linker containing first and second restriction sites between a Lys66 and a Ser67 codon of a barnase gene;
- b. using said first and second restriction sites of said linker to operationally insert a ubiquitin gene between two amino-acid codons of said linker, thereby creating a ubiquitin-barnase fusion gene;
- c. fully sequencing said ubiquitin-barnase fusion gene to verify its integrity;
- d. using enzymes to operationally insert said ubiquitin-barnase fusion gene into any plasmid of a BL21 (DE3) family, thereby creating an interim ubiquitin-barnase fusion expression plasmid;
- e. operationally inserting a gene for barstar and its natural promoter from *Bacillus amyloliquefaciens* into said interim ubiquitin-barnase fusion expression plasmid, thereby creating a ubiquitin-barnase fusion-barstar complex plasmid;
- f. cloning said gene for barstar into a T7 promoter-containing plasmid conferring resistance to an antibiotic other than ampicillin onto cells transformed by said T7 promoter-containing plasmid, thereby creating a barstar plasmid;
- g. transforming *E. coli* BL21 (DE3) cells grown at about 20 to 37 degrees C in any medium compatible with *E. coli* growth using both said barstar plasmid and said ubiquitin-barnase fusion-barstar complex plasmid, and inducing said *E. coli* BL21 (DE3) cells with about 100 mg/L isopropyl b-D-thiogalactopyranoside;
- h. harvesting said transformed *E. coli* cells by centrifugation after about 2 to 12 hours; after said induction;
- i. placing said harvested *E. coli* cells in 10 mM sodium phosphate at a pH of 7.5, thereby creating a solution of harvested *E. coli* cells;
- j. lysing said solution of harvested *E. coli* cells by repeated freeze-thaw cycles in the presence of about 10mg/liter lysozyme, thereby creating a lysate;

- k. adding about 10 mg/liter DNase I to reduce the viscosity of said lysate;
- l. centrifuging said reduced viscosity lysate to remove insolubles, thereby forming a supernatant;
- m. adding about 8 M urea to said supernatant to dissociate bound barstar;
- n. Removing said dissociated barstar from said supernatant by passing said supernatant through an anion exchange chromatography resin to yield a solution;
- o. loading said solution onto a cation exchange column;
- p. washing said solution with about 10 mM sodium phosphate (pH about 7.5) and about 6 M urea;
- q. eluting said solution using a 0 to 0.2 M NaCl gradient;
- r. Removing said urea from said dilution by dialysis against double-distilled water to yield barnase-ubiquitin fusion protein.